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(54) Title: RECOMBINANT PLANT ENZYME

(57) Abstract

Disclosed is a nucleotide sequence encoding an enzyme having acyl-ACP thioesterase activity comprising nucleotides 169-1269 of the sequence shown in Figure 1 or functional equivalents thereof. Also disclosed is a polypeptide possessing said enzyme activity, vectors, host cells, and transgenic plants comprising the novel nucleotide sequence, together with a method of altering the characteristics of a plant and a method of producing the enzyme.

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Title: Recombinant Plant Enzyme

Field of the Invention

This invention relates to the novel nucleotide sequence of a gene encoding a plant enzyme, functional equivalents thereof, vectors containing the novel nucleotide sequence, a method of producing the enzyme, a method of altering the characteristics of a plant and plants having been so altered.

Background of the Invention

Many plants and plant-derived materials (eg sunflowers, oil seed rape) are of commercial significance because they are a source of valuable vegetable oils. These oils (or lipids) consist of fatty acid residues (relatively long hydrocarbon chains) joined by ester links to propan -1, 2, 3 - triol (otherwise known as glycerol).

In some higher plants, de novo fatty acid biosynthesis is known to be catalyzed by two enzymes, acetyl-CoA carboxylase and fatty acid synthetase. The end product of de novo fatty acid biosynthesis is palmitoyl-ACP (Acyl Carrier Protein) which is then rapidly elongated to stearoyl-ACP. A highly specific stearoyl-ACP desaturase ensures that the only unsaturated fatty acid in high abundance is oleoyl-ACP. Another enzyme, acyl-ACP thioesterase is thought to play an important role in the chain termination of fatty acid biosynthesis by catalysing

the hydrolysis of acyl-ACP to free fatty acid and ACP. It is believed that subsequently the free fatty acid is incorporated into complex lipids.

Acyl-ACP thioesterases may be characterised by their preferred substrates. Thus acyl-ACP thioesterases showing preferential enzymatic activity for long chain fatty acids (eg C18 compounds) may be termed "long chain thioesterases".

Some long chain acyl-ACP thioesterases from plants have been identified and purified. These include the enzymes from Avocado and Safflower (Ohlrogge et al., [1978] Archives of Biochemistry and Biophysics 189, 382-391 and McKeon & Stumpf, [1982] Journal of Biological Chemistry 257, 12,141-12,147, respectively). However, no-one has been able to obtain the DNA sequence encoding such a long chain thioesterase. Such information would allow the application of modern recombinant DNA techniques to manipulate the enzyme characteristics (eg level of expression), and hence the characteristics of transgenic plants.

Summary of the invention

In one aspect the invention provides a nucleotide sequence, encoding an enzyme precursor having an acyl-ACP thioesterase activity, comprising the sequence of nucleotides 169-1269 shown in Figure 1 (Seq. ID No. 1) or functional equivalents thereof.

As will be apparent to those skilled in the art, functional equivalents of the nucleotide sequence of the invention include, for example: those nucleotide sequences

which encode the same polypeptide with the same activity (i.e. which cleave acyl-ACP molecules) but which, by virtue of the degeneracy of the genetic code, possess a different nucleotide sequence; sequences which encode substantially the same polypeptide but wherein there may be one or more conserved amino acid substitutions (i.e. the substitution of an amino acid for one with similar properties); sequences which encode substantially the same polypeptide (which preferably share at least 50% amino acid homology and more preferably at least 60% homology) but wherein there may be one or more minor deletions or truncations; and sequences which hybridize under standard conditions to the complement of nucleotides 169-1269. Typically such functional equivalents will have at least 75% nucleotide sequence homology and preferably at least 85% homology. An example of a functional equivalent is the sequence pNL3 (Seq. ID No. 3) shown in Figure 2.

A particular example of a functional equivalent is the sequence comprising the antisense equivalent to the sequence of the invention. Whilst antisense sequences are not generally understood to be functional equivalents, use of the term functional equivalent is intended for the purposes of the present application to encompass such sequences.

Another particular example of functional equivalents are those sequences which code for the mature enzyme rather than the enzyme precursor. Studies described below show that the precursor includes an N-terminal transit peptide of about 4kDa. Thus the N-terminal 27-37 amino acids (preferably the N terminal 29-35 amino acids) could be omitted. Alternatively, the transit peptide could be exchanged for the transit peptide of another protein (e.g.

that of ACP).

Preferably the sequence also comprises a suitable 5' untranslated region, including a promoter, to enable expression in appropriate host cells.

Preferably the sequence also comprises a suitable 3' untranslated region. As well as a stop codon, this 3' untranslated region can comprise other signals, such as a polyadenylation signal.

In another aspect, the invention provides an enzyme precursor having an acyl-ACP thioesterase activity, comprising the amino acid sequence shown in Figure 1 (Seq. ID No. 1) or functional equivalents thereof.

As described previously, functional equivalents can include mature enzymes lacking the N terminal transit peptide (which is thought to be cleaved between Ala33 and Val 34, or in the vicinity thereof). Other functional equivalents include chimaeric polypeptides comprising the transit peptides of other proteins (such as that from ACP).

An example of such an enzyme precursor is the acyl-ACP thioesterase from Brassica napus (oil seed rape), the gene for which has been cloned and sequenced by the inventors. The sequences of the full length cDNA clones obtained, pNL2 and pNL3, are shown in Figures 1 and 2. A number of sequence variations can be seen (detailed in Figure 3). Some of these nucleotide sequence differences result in different deduced amino acid sequences, as shown in Figure 4.

It is thought that all these sequence variations are manifestations of the same phenomenon: it is believed that the B. napus acyl-ACP thioesterase enzyme is encoded by a multi-gene family (i.e. there are a number of acyl-ACP thioesterase allelic genes in B. napus). This has already been demonstrated for the B. napus ACP gene (Safford et al., (1988), European Journal of Biochemistry 174, 287-295). There is also evidence to suggest that there are several (10-20) copies of the thioesterase gene in the B. napus genome (see Figure 6).

Thus in a specific embodiment the invention provides a nucleotide sequence encoding an enzyme precursor having acyl-ACP thioesterase activity and comprising the amino acid sequence shown in Figure 1, or functional equivalents thereof.

In another aspect, the invention provides a vector containing a nucleotide sequence in accordance with the invention.

A further aspect of the invention comprises a cell transformed with the vector defined above and thus capable of expressing an enzyme precursor with acyl-ACP thioesterase activity.

Such a transformed cell may be of bacterial, fungal, plant or animal origin.

Such transformed cells can be grown in cultures using methods well known to those skilled in the art. Thus, in the case of a transformed plant cell, one may obtain a transgenic plant.

In yet another aspect, the invention provides a method of producing an enzyme precursor having acyl-ACP thioesterase activity comprising the steps of: inserting a sequence encoding the enzyme precursor or a functional equivalent into a suitable expression vector; transforming a suitable host cell with said vector; growing said transformed host cell in suitable culture conditions; and obtaining the enzyme precursor from the host cells and/or from the culture medium.

Preferably, a sequence is employed which encodes a functional equivalent so as to produce the mature enzyme.

In view of the disclosures made herein, suitable vectors, host cells and culture conditions will be apparent to those skilled in the art.

It may be advantageous to obtain the enzyme precursor free from contamination by other plant proteins or products, thus in a preferred embodiment the enzyme precursor is expressed in an animal or bacterial cell. Most preferably a microorganism is used to express the enzyme precursor. Conveniently the precursor is secreted.

The invention has a number of applications. It might prove possible, by under- or over-expressing this enzyme precursor (or mature enzyme), to alter the properties of the storage lipid molecules of the plant in which the enzyme precursor is expressed. For instance, by over-expressing the enzyme precursor it might be possible to bring about premature termination of the hydrocarbon chain elongation during fatty acid biosynthesis, thus resulting in lipids with shorter hydrocarbon chain fatty acid components. Such "medium chain" lipids are useful as

"hardening stock" in the production of margarines and detergents. Alternatively, by use of an anti-sense construct, it might be possible to reduce the levels of active enzyme in the plant, resulting in the increased production of longer chain fatty acids. Equally, because the enzyme catalyses the last stage in fatty acid biosynthesis, it should prove possible to modify the fatty acid levels of the plant.

Typically the plants to be altered could include rape, sunflower, safflower, soybean, peanut, cotton, oil palm or corn.

Thus in another aspect the invention provides a method of altering the characteristics of a plant, comprising introducing into the plant the sequence of the invention or a functional equivalent thereof, so as to alter the level of acyl-ACP thioesterase activity.

Preferably the enzyme precursor or function equivalent is selectively expressed in the plant seeds. This can be achieved by expressing the precursor or functional equivalent under the control of a seed specific promoter. Several such promoters are known (e.g. those for the ACP, napin and cruciferin genes).

The characteristics one might expect to alter, in addition to acyl-ACP thioesterase activity are those influenced by levels of the enzyme precursor or mature enzyme, such as fatty acid yield and composition.

Preferably the characteristic altered is the storage oil yield or composition of the seeds of the plant.

The acyl-ACP thioesterase from B. napus exhibits a strong substrate specificity, with oleoyl-ACP (18:1-ACP) being the preferred substrate. Ideally, the product hydrocarbon should have chain lengths shorter than 18 carbon atoms. One plant which contains storage lipids with fatty acid components of the preferred length is Cuphea. Thus, by use of DNA probes based on knowledge of the B. napus enzyme sequence, and by use of antibodies raised against the purified B. napus enzyme, it might be possible to identify clones containing the sequence encoding the corresponding Cuphea enzyme, which could be used to modify fatty acid biosynthesis in the desired manner.

The invention is further described by reference to the following examples and drawings of which:

Figure 1 shows the DNA sequence of cDNA clone pNL2 which encodes the B. napus acyl-ACP thioesterase gene and the deduced amino acid sequence of the enzyme,

Figure 2 shows the nucleotide sequence of cDNA clone pNL3 which encodes an allelic variant of B. napus acyl-ACP thioesterase, and the deduced amino acid sequence,

Figure 3 shows the variations in nucleotide sequence between pNL2 and pNL3,

Figure 4 shows the variations in the deduced amino acid sequence of the proteins encoded by pNL2 and pNL3

Figure 5 shows the amino acid sequences obtained by direct sequencing of peptides generated by enzymatic digestion of B. napus acyl-ACP thioesterase, with boxes indicating residues which show variation from the amino acid sequence

expected from the cDNA sequences, and

Figure 6 shows a B. napus genomic Southern blot probed with an oilseed rape thioesterase probe.

Example 1

Figures 1 and 2 show the nucleotide sequence of pNL2 and pNL3 respectively, full length cDNA clones of the B. napus acyl-ACP thioesterase gene, together with the deduced amino acid sequences of the respective proteins encoded. Several differences are observed between the amino acid sequence obtained directly from the protein and that deduced from the cDNA clones (see Figure 5).

Clones pNL2 and pNL3 were obtained in the following manner.

Poly A^+ RNA was isolated from 25-30 days after flowering (D.A.F.) B. napus embryos, as previously described [Hall et al. (1978), Proceedings of the National Academy of Sciences 75, 3196-3200], and used to construct a lambda gt10 cDNA. The cDNA was made essentially according to the manufacturer's instructions (Pharmacia) and then ligated into the vector and packaged in vitro. Basically, double stranded oligo dT-primed cDNA was synthesised from poly A^+ RNA according to Gubler and Hoffman [Gene (1983) 25:203-269]; EcoRI linkers were ligated to the ends, and the resulting material cloned into a bacteriophage vector, lambda gt10 (Amersham International).

Approximately 50,000 clones were screened using an oligonucleotide probe (RAAT 3AS - see below, Seq. ID No. 5), derived from the rape thioesterase peptide VMMNQDT.

Several positive clones were obtained and the longest (1200bp) was subcloned into pTZ18R (U.S.B.) making a construct termed pNL1. Following sub-cloning into MI3, overlapping fragments of pNL1 were sequenced in both directions.

```
                                  A
                A  T  A           C
RAAT 3AS 5' GTGCCCTGGTTCAATCATGAC 3' 21 mer
                                  T
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Translation of the DNA sequence gave rise to an open reading frame (ORF) of 233 amino acids plus a 325bp 3' non-coding sequence. The ORF contained a number of sequences that had been identified by direct amino acid sequencing of rape acyl-ACP thioesterase peptides. pNL1 was therefore determined to code for rape acyl-ACP thioesterase.

A second B. napus cDNA embryo library was constructed in lambda ZAPII (Stratagene), according to the manufacturer's protocol. 100,000 plaques were screened with a 730bp BglII pNL1 fragment and eight positive clones were obtained. The eight clones were purified to homogeneity and in vivo excised, according to the Sratagene protocol, to give rescued plasmids containing the cDNA insert within the polylinker. Restriction analysis revealed that two plasmids, pNL2 and pNL3, contained inserts of 1560bp and 1460bp respectively. pNL2 and pNL3 were subcloned into M13 and sequenced. Translation of the resulting DNA sequences showed both pNL2 and pNL3 to contain ORFs of 363 amino acids, but differing in the length of their 3' non-coding sequences.

Neither pNL2 nor pNL3 contained an initiating methionine and, in order to obtain this, the technique of 5' RACE (Rapid amplification of cDNA ends) was employed, essentially as described by Frohman et al. [Proceedings of the National Academy of Sciences 75, 8998-9002]. 1ug of 25-30 D.A.F. B. napus embryo poly A^+ RNA was reverse transcribed at 37°C for 60 min with 200 units M-MuLV Superscript reverse transcriptase (BRL) and 500 pmoles random hexamers as primer. Excess primers were removed on a Centricon 100 column and cDNA precipitated with isopropanol. After resuspension in 10ul TE buffer, the cDNA was tailed using 10 units terminal transferase (BRL) in buffer (BRL) and 200uM dATP at 37°C for 10 min. After treatment at 65°C for 5 min the mixture was diluted to 500 ul with TE. 10ul mixture was used in a 100ul second strand Polymerase Chain Reaction (PCR) with 50 pmol rape thioesterase specific primer PTB5
[5' GATTAGCAATTGTCTCGACGG, Seq. ID No. 6],
50-pmol R primer
[5' AAGGATCCGTCGACATC Seq. ID No. 7]
and 5pmol T_{17}-R_0-R_I primer
[5' AAGGATCCGTCGACATCGATAATACGACT
CACTATAGGGATTTTTTTTTTTTTTTTT, Seq. ID No. 8].
The mixture was heated to 95°C for 7 min then held at 72°C. 10ul 10 x PCR buffer (BRL), 200uM dNTPs and 2.5 units Taq polymerase were added, heated at 45°C for 2 min then 72°C for 40 min. To PCR amplify the products, the mixture was heated to 94°C for 45 min to denature cDNA, primer annealed at 50° for 25 min and extended at 72°C for 3 min. This cycle was repeated 34 times, followed by final extension at 72°C for 10 mins.

1ul of the above PCR products was used in a second amplification containing 200uM dNTPs, 2.5 units Taq, 50

pmole R_1
[5' GACATCGATAATACGAC, Seq. ID No. 9],
50 pmole rape thioesterase specific primer PTB2
[5' CAAGAATTAACAGGGCTGATG, Seq. ID No. 10],
(internal to PTB5), PCR buffer added to 50ul. Mixture heated 94° for 45 min, 50°C for 30 min and 72°C for 1 min - 28 cycles followed by final extension at 72° for 10 min. The products of this second PCR amplification, after size selection on agarose gels, were ligated into pT7 Blue (AMS Biotechnology). Recombinants were identified by PCR using 50 pmol R_1 primer and 50 pmol PTB3, another rape thioesterase specific primer [5' GCGATTAACCGGGATGAAAAG, Seq. ID No. 11]
internal to PTB2. This mixture was heated to 94°C for 1 min, 55°C for 1 min, 72°C 1.1/2 min. This cycle was repeated 24 times and the products subjected to a final extension at 72° for 10 min.

144 colonies were screened and 15, containing the largest inserts, were selected for DNA sequence analysis. The 2 longest inserts, RACE 6 and RACE 1, were both found to be 180bp longer than the 2 cDNA clones pNL2 and pNL3 at the 5' end and to contain a putative initiating methionine. A 160bp overlapping region between the cDNAs and the RACE products enabled the origin of the 2 RACE products to be determined. Thus RACE 6 was identical in sequence to pNL2 and RACE 1 was identical to pNL3.

The complete nucleotide and deduced amino acid sequences of pNL2 and pNL3 are shown in Figure 1 and Figure 2 respectively. The 2 composite clones pNL2 and pNL3 represent entire copies of the structural genes of rape acyl-ACP thioesterase. Excluding poly(A) tails, pNL2 contains 1642bp and pNL3 1523bp. Both pNL2 and pNL3 have

putative initiation methionines at nucleotide 169, which show good homology to the plant consensus translation initiation sequence, and to encode open reading frames (ORFs) of 366 amino acids. The two clones differ in the length of their 3' non-coding sequences. The open reading frames of pNL2 and pNL3 encode polypeptides of 41,960 Da and 41,983 Da respectively and these represent the precursor form of the thioesterase. On SDS-PAGE, mature rape thioesterase migrates with a molecular weight of 38 kDa (see below) suggesting a transit peptide of about 4 kDa. Since the N-terminus of the mature protein is blocked to Edman degradation the precise cleavage site of the precursor polypeptide could not be determined.

Figure 3 shows the nucleotide sequence variation between cDNA clones pNL2 and pNL3. Some of these sequence differences are observed to be in the ORFs, and result in different deduced amino acid sequences for the polypeptides encoded by pNL2 and pNL3. These differences are illustrated in Figure 4.

To further characterise the protein, it was highly purified, as described below. (All steps were carried out at 4°C unless otherwise stated.)

Step 1: Preparation of cell free extract

Rape seed (100g), which had been harvested between 40-60 days post anthesis (after flowering), and stored at -70°C, was homogenised in 250ml 20mM potassium phosphate, pH 7.3, containing 2mM dithiothreitol and 0.2mM phenylmethylsulfonyl fluoride using a polytron at maximum speed. The resulting homogenate was stirred for 30 minutes with Triton X-100 (stock solution 20% v/v) at a

final concentration of 1% v/v. After centrifugation at 20,000g for 30 minutes the supernatant was filtered through glass wool to remove the floating lipid layer.

Step 2: Ammonium Sulphate fractionation

The filtrate was brought to 30% saturation by the addition of solid ammonium sulphate and the suspension was stirred for 30 minutes prior to centrifugation at 20,000g for 30 minutes. The supernatant was refiltered through glass wool and brought to 50% saturation with solid ammonium sulphate. After stirring for 45 minutes the extract was centrifuged for 20 minutes at 30,000g. The pellets were stored at -20°C until required.

Ammonium sulphate pellets were resuspended in Buffer A (20mM bis-Tris HCl, pH 6.5, containing 2mM dithiothreitol and 0.2mM PMSF) and centrifuged at 30 000g for 10 minutes to remove particulate matter.

Step 3: Fast Flow Q-Sepharose chromatography

The supernatant was diluted with Buffer A until the conductivity was less than 20 x 10^{-4} ohms (final volume 200ml) and loaded onto a Fast Flow Q-Sepharose column (2.5cm x 12cm) equilibrated in 20mM bis-Tris HCl, pH 6.5. After loading, the column was washed with equilibration buffer until absorbance at 280nm was less than 0.15. Enzyme activity was eluted with a 300ml gradient from 0-0.5M NaCl in Buffer A. Fractions (5ml) were collected and assayed for activity. The flow rate throughout was 1.3ml $minute^{-1}$. The unbound sample was assayed for activity and typically less than 10% of the activity loaded did not bind. Thioesterase activity eluted between 150 and 300mM

NaCl. Fractions containing activity were pooled (115-120ml) and stored overnight at -70°C.

Step 4: ACP-Sepharose chromatography

The Q-Sepharose pooled fractions were rapidly thawed and applied to an ACP-Sepharose column (1cm x 13cm) equilibrated in 20mM potassium phosphate pH 6.5. After loading, the column was washed with 0.1M potassium phosphate, pH 6.5, alone. Activity was eluted with 0.3M potassium phosphate pH 8.0. All buffers contained 2mM DTT and 0.2mM PMSF and the flow rate throughout was 0.5ml $minute^{-1}$. Fractions (5ml) were collected and assayed for activity. Using freshly synthesised affinity matrix, greater than 95% of the activity loaded was bound and no activity was detected in the wash fractions. Typically, 30-40% of the activity loaded was eluted. Fractions containing activity were pooled (25-30ml) and stored at -20°C overnight. Typically 70% of activity was retained after thawing.

Step 5: F.P.L.C Mono P chromatography

The ACP-Sepharose pool was dialysed against 2.5l 25mM bis-Tris, iminodiacetic (pH 7.1) acid for 4.5 hours with one change of buffer after two hours. The conductivity of the dialysed sample was checked and, if necessary, the sample was diluted with 25mM bis Tris pH 7.1 until the conductivity was less than 10×10^{-4} ohms. The sample was loaded via a 50ml superloop onto a Mono P column (HR 5/20) equilibrated in 25mM bis-Tris iminodiacetic acid pH 7.1 at a flow rate of 1 ml $minute^{-1}$ at room temperature. The column was washed with equilibration buffer and activity was eluted with 50ml 10% polybuffer 74 (v/v)/iminodiacetic

acid, pH 4.0, at a flow rate of 1 ml minute^{-1}. Fractions (1ml) were collected into tubes which contained 50ul 1M bis-Tris pH 9.9 to immediately neutralise the elutant since thioesterase activity appeared to be unstable at low pH. To determine the pH elution gradient, the column was calibrated with a blank run prior to loading the enzyme. Under these chromatographic conditions, all activity bound to the column and typically 40-50% of the activity loaded was recovered in five fractions.

Active fractions eluted from the Mono P column were immediately concentrated on an Amicon 10 microconcentrator, pre-coated with protein to prevent non-specific binding. Concentrated sample (final volume 1ml) was brought to 0.2M NaCl and stored at -20°C overnight. Samples stored at -20°C lost almost no activity while those stored at 4°C lost typically 60% activity.

Step 6: F.P.L.C. Superose 12 gel filtration

On thawing, the Mono P concentrated pool was centrifuged on an MSE centaur for 5 minutes prior to loading onto two Superose 12 gel filtration columns HR10/30 (1 x 30cm) in series. To obtain maximum purification, care was taken not to overload the column and therefore 200ul aliquots of the supernatant were loaded for each run. The column running buffer was 50mM sodium phosphate, pH 7.2, containing 150mM NaCl. Fractions (200ul) were collected and assayed for activity. Activity eluted as one peak over 5-6 fractions and typically 45% of the activity loaded was recovered. Five runs were necessary to purify the concentrated sample.

Once partially purified, the enzyme was further

characterised. The intact enzyme was found to have a molecular weight of about 70 kDa, being composed of a dimer of approximately 38kDa sub-units. A degraded form of the enzyme (33kDa) was found to co-purify with the intact 38kDa subunits. The pH optimum was 9.5. As with other plant acyl-ACP thioesterases so far described, the preferred substrate of the B. napus enzyme was found to be oleoyl-ACP (18:1-ACP).

The partial amino acid sequence of the purified protein was determined as described below.

The highly purified preparation, after Mono P chromatography, was concentrated and reductively alkylated with 4-vinyl-pyridine. Protein samples were loaded directly, via a 100ul loop, onto a Brownlee C-8 (CO3-032) column pre-equilibrated in 0.1% TFA (v/v). Flow rate was 0.1ml min^{-1}. The column was washed with 0.1% TFA until absorbance at 214nm was close to baseline. Protein was eluted with a gradient of increasing Buffer B (90% acetonitrile; 0.085% TFA) 0-70% over 35 min. It was necessary to pause the gradient for 40-45 min at 38% Buffer B to elute isocratically a major absorbance peak which contained no protein and was probably a contaminant from 4-vinyl-pyridine. Protein peaks were collected manually into Eppendorf tubes, fractions containing acyl-ACP thioesterase polypeptides (38 and 33kDa) were pooled.

After reversed-phase chromatography, the pooled purified enzyme preparation was brought to near dryness on a Univap concentrator. The appropriate digestion buffer, detailed below, was added and the pH was measured using indicator paper and adjusted with 1M NaOH. During the concentration step, a heavy precipitate formed which disappeared on

neutralisation. The final volume of samples prepared for digestion was 100-150ul.

Preparations containing 3-7ug (80-180 pmoles) of purified thioesterase polypeptides (38 and 33kDa) were digested with 0.15ug Endoproteinase Lys-C (Boehringer Mannheim). To check for auto-digestion a control sample was prepared with 0.15ug protease in 150ul 100mM Tris/HCl (pH 8.5), 2mM EDTA (digestion buffer). Incubations were left at 37°C for 10-16 hours. Additional Endoproteinase Lys-C (0.15ug) was added to the thioesterase and control incubations. Digestion was allowed to proceed at 37°C for 40 hours. Samples were stored at -20°C prior to reversed-phase chromatography.

Similarly a preparation, containing 9ug (240 pmoles) of purified enzyme (38 and 33kDa polypeptides), was digested with 0.18ug TPCK-typsin (Cooper Biomedical) overnight at 37°C. A control sample was prepared with 0.18ug TPCK-trypsin in 100ul 0.2M ammonium carbonate/1mM calcium chloride (digestion buffer) and incubated as for the protein sample. Fresh TPCK-trypsin was prepared and a further 0.18ug was added to the protein and control incubations. Both samples were left at 37°C for 8 hours. Samples were stored at -20°C prior to reversed-phase chromatography.

Reversed-phase chromatography was performed at room temperature on Gilson HPLC equipment adapted for microbore column chromatography. Samples were loaded, via a 100ul loop, onto an Aquapore RP 300-C8 microbore column (250 x 1mm id; 7u) pre-equilibrated in 0.1% TFA. Flow rate was 0.1ml/minute. Peptides were eluted with a gradient of increasing buffer B (90% acetonitrile; 0.085% TFA) 0-70%

over 100 minutes. Absorbance was monitored at 214nm and peaks were collected manually into Eppendorf tubes. Prior to loading sample, acetonitrile gradients were performed until a reproducible low baseline was obtained.

N-terminal Protein Sequencing was performed on an Applied Biosystems model 475 protein sequencer as described previously (Cottingham et al., [1988] Biochim. Biophys. Acta 954, 201-207). A number of peptide sequences were obtained. These are shown in Figure 5 and are Seq. ID Nos 12 (peptide 26), 13 (peptide 15), 14 (peptide 30), 15 (peptide 34) and 16 (peptide 39). The residues boxed in the tryptic peptides are different to those expected from translation of the cDNA sequences. These differences are thought to be due to the allelic nature of the gene.

From the partially determined amino acid sequence, it was possible to identify nucleic acid sequences encoding the enzyme. The techniques and methods of accomplishing this are well known to those skilled in the art. Figure 5 shows a genomic Southern blot probed with thioesterase - specific probe. Molecular standard markers (obtained by a PstI digest of lambda DNA) are shown on the right hand side with their size shown in kilobases. The middle four lanes represent controls having a known copy number (1, 2, 5 or 10) whilst Hpa, Sac and Bam are restriction digests of rape genomic DNA resulting in 12, 17 and 16 copies respectively per haploid genome. The probe used was a 764bp fragment generated by PCR, using primers PTB1 AS and PTB2AS (Seq. ID Nos 17 and 18 respectively), shown below:

PTB1AS 5' CCGAGCTCGTCGATTGATGGAAGA 3' 24-mer 201-224bp

PTB2AS 5' TTGGTACCGGTAAGCTTTGAGATC 3' 24-mer 942-965bp

Example 2

This example describes the construction of a plant transformation vector containing an antisense rape thioesterase fragment linked to a seed-specific acyl carrier protein (ACP) promoter for use in altering fatty acid composition and oil yield in transgenic oil seeds.

The 764 bp fragment of pNL2 (described above) was produced by PCR amplification using the primers PTB AS1. PTB AS1 and PTB AS2 incorporated SacI and Kpn1 restriction sites respectively into the fragment. A 100ul PCR reaction contained 10ul 10x reaction buffer (Stratagene), 200uM dNTPs, 50 pmole PTB AS1, 50 pmole PTB AS2, 100pg pNL2, 2.5 units Taq (Stratagene). Reaction conditions were 92° for 45 sec, 55°C for 45 sec, 72°C for 90 sec for 25 cycles followed by 72°C for 10 min. The 764 bp fragment was recovered via DEAE paper from agarose gels, digested with SacI and Kpn I, re-run on agarose gels and recovered from DEAE paper.

A 1.4kb BamHI-BglII fragment of pTZ5BS [de Silva et al., (1992) Plant Molecular Biol. 18:1163-1172], representing a seed specific ACP promoter sequence, was ligated into BamHI-restricted pTZ18R [Mead et al., (1986) Prot. Engin. 1: 64-74] to produce pTZAP1. pTZAP1 was restriction analysed to confirm correct orientation of ACP promoter fragment. pTZAP1 was restricted with SacI and Kpn1 and recovered from agarose gel via DEAE paper. The recovered pTZAP1 was ligated with the 764 bp NL2 fragment from above to form pNL4. pNL2 and the plant transformation vector pBI 101 (Clontech) were digested with BamH1 and Sac1. The 2.15kb fragment of pNL2 and the pBI 101 vector fragment

were recovered from DEAE cellulose and ligated to form pETR1. pETR1 was subjected to restriction analysis to confirm correct insertion of gene fragments.

Example 3

Agrobacterium-mediated Transformation of Brassica napus

The vector pETR1 was transferred into Agrobacterium tumefaciens pGV3850 (Zambryski et al., (1983) EMBO J. 2:2143-2150) using a direct DNA uptake procedure [An et al., (1988) In Plant Molecular Biology Manual (Gelvin & Schilperoort, Eds.), Kluwer Academic Publishers, pp. 1-19]. From the resultant Agrobacterium colonies, DNA was extracted, transformed into E. coli from which it was re-isolated to confirm correct gene insertion.

Brassica napus (cv. Westar) stem segments were transformed with the above binary Agrobacterium strain using the procedure of Fry et al. [(1987) Plant Cell Reports 6: 321-325] with the following modifications: kanamycin selection was at 20ug/ml and was delayed until 2 weeks after infection; carbenicillin was replaced by cetotaxime (500ug/ml); arginine was omitted from the regeneration media; 0.8% agar was replaced by 1% agarose; a 2-3 day pre-treatment of stem segments was carried out prior to Agrobacterium infection and a N. plumbaginifolia cell line [Barfield et al. (1985), Plant Cell Reports, 4: 104-107] was used as feeder layer. Shoots formed and remaining green on Plant Cell Reports, 4: 104-107] was used as feeder layer. Shoots formed and remaining green on selective media after 2 transfers were tested for the presence of nopaline [Otten and Schilperoort (1978), Biochem. Biophys. Acta, 527: 497-500]. Positive shoots

were transferred to soil, potted on into 5" diameter pots and transferred to growth rooms operating a 16h day (22°C) and 8h night (18°C) cycle.

Control plants were transformed with A. tumefaciens pGV3850 containing pAPIGUS [de Silva et al. (1992), Plant Molecular Biology, 18: 1163-1172], a plasmid in which the ACP promoter controls the expression of the beta-glucuronidase (GUS) reporter gene.

Example 4

Analysis of Transformed Plants

DNA was extracted from pETR1 antisense thioesterase plants and from APIGUS transformed control plants and Southern blotted to confirm the presence of inserted genes.

Mature seeds from 39 pETR1 transformed plants and 10 APIGUS transformed plants were analysed for oil content and for fatty acid composition.

After weighing, seeds were macerated in methanol, using an Ultraturrax homogeniser, and extracted overnight in chloroform/methanol (2:1 v/v). After removing seeds by filtration, samples were partitioned with 1% sodium chloride solution. The chloroform-soluble lipid fraction was removed and evaporated to dryness. To analyse for fatty acid composition, extracted lipids from above were refluxed for 60 min with acid methanolysis reagent (toluene/methanol/conc. sulphuric acid 10:20:1). To determine the total fatty acid content of the samples heptadecanoic acid (C17:0) was added as an internal standard (to about 20% of total lipid weight). After

addition of water, the resultant fatty acid methyl esters were extracted with hexane and analysed by gas chromatography on a Carlo Erba chromatograph equipped with a Supelcowax 10/fused silica column.

Table 1 shows the percentage total fatty acid (% TFA) content and the fatty acid composition of seeds from the transgenic plants. The fat content of the control APIGUS seeds (shown as AP1-TAK in Table 1) is seen to range from 38.1%-27.3%. The fat content of the pETR1 antisense thioesterase seeds ranged from 43.2%-14.7%. The reduced oil content of the pETR1 seeds could result from reduced levels of thioesterase activity. Acyl-ACP thioesterase serves as the chain terminating enzyme of de novo fatty acid biosynthesis, cleaving the synthesized acyl-ACP to free fatty acid and ACP. As such, decreasing the level of this terminating enzyme, via antisense technology, could have limited the overall rate of oil synthesis in the seed.

In respect of fatty acid composition, 2 pETR1 plants, 19 and 9, have altered fatty acid compositions of potential commercial use. Seeds from these plants both contain dramatically reduced levels of linolenic acid (18:3) and also significantly reduced levels of linoleic acid (18:2). The production of rape oils containing reduced polyunsaturated fatty acids, such as those from plants 19 and 9, is highly desirable for use as cooking oils since they are less susceptible to oxidation.

Example 5

This example describes the construction of plant transformation vectors containing sense rape thioesterase

genes linked to the seed specific ACP promoter for use in altering fatty acid yield and composition in transgenic oil seeds.

To modulate fatty acid biosynthesis within cells of seed tissue by "overexpressing" rape thioesterase, it is necessary to target the thioesterase enzyme to the intracellular site of fatty acid synthesis, namely the plastid. Prior to obtaining a full length thioesterase cDNA, which contained a plastid targetting sequence, a chimaeric thioesterase gene was constructed in which a rape ACP plastid transit sequence was fused to a partial thioesterase cDNA which encoded for the mature enzyme. The chimaeric thioesterase gene was linked to the seed specific rape ACP promoter.

A 361 bp fragment of pNL2, encoding the N-terminal region of the rape thioesterase, was produced from pNL2 by PCR using the primers PTB 26
(5' CTTCTAGATCTCAATGTGCATT, Seq. ID No 19) and PTB 33
(5' CCGAATTCTGCAGTCTCGTCTTCTC, Seq. ID No 20).
PCR reaction (100ul) contained 10ul 10x reaction buffer (Stratagene), 200uM dNTPs, 50 pmole PTB 26, 50 pmole PTB 33, 100ng pNL2, 2.5 units Taq (Stratagene). Mixture heated 92°C for 30 sec, 55°C for 30 sec, 72°C for 60 sec for 25 cycles, followed by 72°C for 10 min for final extension. The PTB 26 primer introduced a Xbal restriction site into the fragment and the PTB 33 primer introduced EcoRI and Pst1 sites. Following isolation from agarose gels and DEAE paper the PCR fragment was restricted with EcoR1 and Xbal, ligated into M13 and DNA sequenced to ensure correct sequence. An EcoRI - BglII version of the PCR fragment was ligated into pNL6 to yield pNL11. pNL6 was constructed by restricting pNL2 with

EcoRI and BglII (partial) recovering the 1.2kb fragment, via DEAE paper; and ligating into EcoRI/BamH1 restricted pTZ19.

A 166 bp ACP transit sequence was obtained from rape ACP cDNA clone 29C08 [Safford et al. (1988), Eur. J. Biochem. 174: 287-295] by PCR amplification using the primers PTB 31
(5' AATGTCGACCACTTTCTGCTC, Seq. ID No 21) and PTB 32
(5' TCGAGCTCTGCAGCGCAGGAGAC, Seq. ID No 22).
PTB 31 introduced a SalI site into the fragment and PTB 32 introduced Sac1 and Pst1 sites. The PCR product was recovered from agarose gel, via DEAE paper, restricted with Sal1 and Sac1 and cloned into M13 to confirm correct DNA sequence. The SAl1-Sac1 fragment was ligated into SalI/SacI restricted pBluescript SK^+ (Stratagene) to form pNL12.

To link the ACP transit sequence to the mature thioesterase sequence pNL11 was restricted with Pst1 and a 1.1kb fragment recovered and ligated into Pst1 restricted, phosphatased pNL12. This produced pNL13.

The 1.4kb ACP promoter (API) was released from pTZ5BS by restricting with BamH1 and Sal1 and was ligated into BamH1/Sal1 restricted pUCBM20 (Boehringer-Mannheim) to yield pNL14.

To link the ACP promoter to the chimaeric ACP-mature thioesterase sequence pNL13 and pNL14 were both restricted with Sal1. A 1.2kb fragment was recovered from pNL13 and ligated into the linearised, phosphatased pNL14 vector to yield pNL15. Following restriction analysis to confirm the correct gene orientation, pNL15 was restricted with

BamH1 and Sac1 to release a 2.9kb fragment containing the ACP promoter, the ACP transit sequence and the mature thioesterase sequence. This fragment was ligated into the plant transformation vector pBI101 which had been restricted with BamH1 and Sac1 to remove the b-glucuronidase (GUS) fragment. This final plasmid, pARTE1, was propagated in E.coli and transferred into A.tumefaciens pGV3850 via the previously described direct DNA uptake method.

The resulting A. tumefaciens strain was used to transform B. napus stem segments as described in Example 3 above. From the resulting transgenic plants, seeds will be analysed for altered thioesterase levels and for altered oil content and fatty acid composition.

Example 6

Upon obtaining a full length rape acyl-ACP thioesterase cDNA, a plant transformation vector was constructed containing this cDNA linked to the ACP promoter-sequence.

Following EcoRI and partial XbaI restriction of pNL2 1.2kb fragment was recovered onto DEAE paper and ligated into pBluescript SK^+ to yield pNL16. A 180bp fragment containing 5' non-coding thioesterase sequence plus 3 amino acids of the transit peptide was obtained by PCR amplification of pNL5, a rape thioesterase genomic clone, using primers PTB 34
(5' TTG TCG ACA CAC ATG CGT CAT, Seq. ID No 23) and PTB 35
(5' GCA AGC TTC AAC ATT TTT GAT, Seq. ID No 24).
Primer PTB 34 added an additional 6 bp of thioesterase sequence onto the RACE 6 product previously described and

also a Sal1 restriction site. PTB 35 introduced a HindIII site into the fragment. The 180bp PCR fragment was recovered from DEAE paper restricted with Sal1 and HindIII and ligated into Sal1/HindIII restricted pBluescript SK^+ to give pNL17. DNA sequence analysis was carried out to confirm correct sequence.

A 440bp fragment containing the transit peptide plus part of the mature thioesterase coding sequence was obtained by PCR amplification of pNL2 using primers PTB 36 (5' ACA AGC TTT CGT GTA ATG TGA, Seq. ID No 25) and PTB 37 (5' GTG AAT TCA GAT CTC AAT GTG CAT T, Seq. ID No 26). The PTB 36 primer introduced a HindIII site 3' to the thioesterase initiating ATG and PTB 37 introduced an EcoRI site 3' to the Bgl II site. The 440bp PCR fragment was recoved via DEAE paper, restricted with HindIII and EcoRI and ligated into HindIII/EcoR1 restricted M13 to yield pM13HENL2. Fragment was sequenced to confirm correct sequence.

pNL17 was restricted with HindIII/EcoRI and ligated to the 440bp HindIII/EcoRI fragment from pM13HENL2 to yield pNL18. A 560bp Sal1-Bg1II fragment was recovered from pNL18 and ligated into pNL16 to form pNL19. pNL19 represents a 'full length' thioesterase cDNA.

To link the thioesterase cDNA to the ACP promoter a 1.4kb Sal1-Sac1 fragment was released from pNL19 and ligated into Sal1-Sac1 restricted pNL14 to yield pNL20. pNL20 was restricted with BamH1 and Sac1 to yield a 2.8kb fragment containing the ACP promoter linked to the thioesterase cDNA. This 2.8kb fragment was ligated into the plant transformation vector pBI101, in which the BamH1-Sac1

fragment encoding the b-glucuronidase (GUS) gene had been removed. The final vector, pRATE, was propagated in E.coli and transferred into A. tumefaciens pGV3850 as previously described.

The resulting A. tumefaciens strain was used to transform B. napus stem segments as described in Example 3. From the resulting transgenic plants, seeds will be analysed for altered thioesterase levels and for altered oil content and fatty acid composition.

Table 1

PLANT NO.	XTFA	C14:0	C16:0	C16:1	C18:0	C18:1 PETR1	C18:1 AP1-TAK	C18:2	C18:3	C20:0	C20:1	C20:2	C22:0	C24:0	C24:1	TRACE
21	43.2	0.0	4.4	0.4	2.9	61.0	58.2	18.3	10.2	0.7	1.1	0.0	0.3	0.1	0.1	0.5
13	39.9	0.0	4.6	0.4	2.2	58.0	61.6	20.6	11.5	0.6	1.2	0.0	0.3	0.1	0.1	0.4
12	39.9	0.1	4.2	0.3	2.3	60.5	61.7	19.4	10.2	0.5	1.2	0.0	0.2	0.0	0.0	0.6
3	38.5	0.0	4.2	0.2	2.1	61.4	61.3	18.8	10.8	0.5	1.0	0.1	0.2	0.0	0.0	0.8
2	37.7	0.1	4.1	0.2	1.9	59.7	59.0	20.6	11.2	0.5	1.0	0.1	0.2	0.0	0.0	0.4
1	37.5	0.1	4.5	0.3	1.8	57.0	59.1	21.8	12.0	0.4	1.0	0.0	0.2	0.0	0.0	0.4
4	37.4	0.1	4.6	0.2	1.6	58.7	62.8	20.9	11.3	0.5	1.0	0.0	0.2	0.0	0.0	0.4
15	36.1	0.1	4.5	0.2	1.6	58.1	53.7	21.6	11.5	0.5	1.0	0.0	0.2	0.0	0.0	0.4
5	34.7	0.1	3.8	0.3	1.6	59.3	49.8	21.4	11.1	0.5	1.0	0.0	0.2	0.0	0.0	0.4
11	34.0	0.0	4.4	0.3	2.2	63.5	48.1	17.3	9.3	0.7	1.1	0.0	0.3	0.0	0.0	0.4
20	32.8	0.1	4.8	0.3	1.8	54.7		21.7	13.1	0.6	1.2	0.0	0.3	0.0	0.0	0.4
9	32.7	0.1	4.6	0.3	2.4	61.6		19.1	9.3	0.7	1.0	0.1	0.3	0.0	0.0	0.4
17	31.2	0.0	4.5	0.3	1.9	61.1		19.0	10.0	0.6	1.2	0.0	0.3	0.0	0.0	0.4
45	28.4	0.0	4.8	0.3	1.5	58.4		21.7	10.1	0.6	1.3	0.1	0.3	0.0	0.0	0.4
25	27.7	0.1	4.2	0.2	1.8	59.5		21.0	9.9	0.6	1.4	0.0	0.4	0.0	0.0	0.4
8	27.5	0.1	4.2	0.2	1.7	61.8		18.9	10.7	0.5	1.1	0.1	0.4	0.0	0.0	0.4
16	26.8	0.0	4.4	0.3	2.2	63.5		17.3	9.3	0.7	1.2	0.0	0.3	0.0	0.0	0.4
40	26.6	0.1	4.6	0.4	1.3	59.6		20.5	10.5	0.5	1.3	0.1	0.4	0.0	0.0	0.4
14	26.4	0.1	5.0	0.3	3.3	61.7		18.2	8.4	0.9	1.0	0.0	0.4	0.0	0.0	0.4
35	26.1	0.0	4.5	0.4	1.4	58.3		21.6	11.1	0.5	1.2	0.0	0.4	0.0	0.0	0.4
7b	26.0	0.1	5.0	0.3	1.8	59.8		20.6	8.6	0.5	1.4	0.1	0.5	0.0	0.0	0.4
38	25.9	0.1	5.3	0.4	1.3	57.5		21.8	10.4	0.5	1.3	0.0	0.4	0.0	0.0	0.4
18	25.3	0.1	4.5	0.3	1.8	56.0		21.8	12.4	0.5	1.2	0.1	0.4	0.0	0.0	0.4
3b	24.6	0.2	5.1	0.4	2.0	60.7		19.0	8.6	0.7	1.5	0.1	0.5	0.0	0.0	0.4
15b	24.5	0.0	4.9	0.2	1.8	64.0		16.8	8.7	0.8	1.4	0.1	0.5	0.0	0.0	0.4
21b	24.2	0.1	4.6	0.3	2.1	61.5		18.8	8.5	0.7	1.5	0.1	0.4	0.0	0.0	0.4
41	24.0	0.1	4.6	0.3	1.9	61.7		19.5	8.5	0.9	1.3	0.0	0.4	0.0	0.0	0.4
6	23.3	0.2	6.2	0.6	3.0	56.5		20.0	10.2	0.8	1.0	0.1	0.4	0.0	0.0	0.4
23	23.2	0.1	5.2	0.4	1.9	62.0		19.3	7.4	0.8	1.4	0.0	0.4	0.0	0.0	0.4
16b	22.8	0.1	5.0	0.4	1.3	56.3		21.9	11.9	0.5	1.3	0.0	0.4	0.0	0.0	0.4
14b	22.5	0.1	5.2	0.4	2.2	60.6		18.8	9.1	0.8	1.4	0.0	0.5	0.0	0.0	0.4
26	21.9	0.0	5.0	0.4	1.4	61.4		18.5	10.0	0.6	1.4	0.0	0.5	0.0	0.0	0.4
7	21.6	0.1	6.1	1.0	1.9	48.2		20.0	11.8	0.8	0.9	0.1	0.4	0.0	0.0	0.4
28	20.5	0.0	4.9	0.3	2.1	62.8		28.0	8.0	1.0	1.5	0.0	0.6	0.0	0.0	0.4
9b	20.4	0.1	6.2	0.7	2.5	66.3		17.7	4.3	0.9	1.5	0.0	0.8	0.0	0.0	0.4
36	18.0	0.0	5.9	0.3	2.1	63.0		15.2	6.4	0.6	1.3	0.0	0.8	0.0	0.0	0.4
46	16.5	0.1	6.1	0.8	1.9	53.2		17.9	9.6	0.9	1.1	0.0	0.4	0.0	0.0	0.4
19	15.7	0.1	5.8	0.4	2.6	69.0		25.1	3.8	1.1	1.5	0.2	0.8	0.0	0.0	0.4
24	14.7	0.2	6.7	0.9	2.0	55.5		12.3	8.0	0.8	1.3	0.0	0.6	0.0	0.0	0.4
10	38.1	0.0	4.6	0.4	2.3		20.2	11.5	0.6	1.3	0.0	0.3	0.1	0.2	0.3	
4	35.7	0.1	4.6	0.3	2.4		17.9	9.7	0.8	1.2	0.1	0.4	0.0	0.1	0.3	
3	34.3	0.0	4.4	0.3	2.2		18.1	10.2	0.7	1.2	0.1	0.4	0.0	0.2	0.3	
6	34.4	0.1	4.3	0.3	2.0		18.5	10.3	0.7	1.3	0.1	0.4	0.0	0.2	0.3	
1	33.7	0.0	4.6	0.3	1.6		19.6	11.6	0.5	1.3	0.1	0.3	0.0	0.2	0.3	
2	32.9	0.1	4.5	0.3	2.2		21.0	10.4	0.6	1.0	0.1	0.3	0.0	0.2	0.3	
7	32.1	0.0	4.4	0.4	2.0		17.9	9.0	0.7	1.3	0.0	0.4	0.0	0.2	0.3	
9	31.0	0.1	4.6	0.3	1.8		23.3	12.8	0.5	1.2	0.1	0.3	0.0	0.2	0.3	
11	29.3	0.1	5.3	0.6	2.0		24.7	13.7	0.6	1.0	0.1	0.3	0.0	0.2	0.3	
8	27.3	0.1	5.4	0.4	1.6		26.7	14.4	0.5	1.2	0.1	0.3	0.0	0.2	0.3	

CLAIMS

1. A nucleotide sequence encoding an enzyme precursor having acyl-ACP thioesterase activity, comprising nucleotides 169-1269 of the sequence shown in Figure 1 (Seq. ID No 1) or functional equivalents thereof.

2. A sequence according to claim 1, comprising nucleotides 169-1269 of the sequence shown in Figure 2 (Seq. ID No 3).

3. A sequence according to claim 1 or 2, further comprising a 5' untranslated region.

4. A sequence according to claim 1, 2 or 3, further comprising a 3' untranslated region.

5. A sequence according to any one of the preceding claims, comprising the sequence obtainable from Brassica napus.

6. A sequence according to any one of the preceding claims, further comprising a seed-specific promoter.

7. A polypeptide having acyl-ACP thioesterase activity, comprising the amino acid sequence shown in Figure 1 (Seq. ID No 1) or functional equivalents thereof.

8. A polypeptide according to claim 7, comprising the amino acid sequence shown in Figure 2 (Seq. ID No 3).

9. A vector comprising a nucleotide sequence according to any one of claims 1-6.

10. A host cell into which has been introduced the sequence of any one of claims 1-6.

11. A plant host cell according to claim 10, which comprises part of, or is capable of giving rise to, a plant.

12. A method of altering the characteristics of a plant, comprising introducing into the plant the sequence of any one of claims 1-6, so as to alter the level of acyl-ACP thioesterase activity.

13. A method according to claim 12, wherein the characteristics so altered comprise the fatty acid yield and/or fatty acid composition of the plant.

14. A method according to claim 12 or 13, wherein the yield and/or composition of seed storage oil of the plant is altered.

15. A method of producing an enzyme having acyl-ACP thioesterase activity, comprising: inserting the sequence of any one of claims 1-6 capable of expressing the enzyme into a suitable expression vector; transforming a host cell with said vector; growing said transformed host cell in suitable culture conditions; and obtaining the enzyme from the host cells and/or the culture medium.

16. A method according to claim 15, wherein the host cell is a microorganism.

RAPE ACYL-ACP THIOESTERASE CLONE pNL2

```
                    v10         v20         v30         v40
          GGCGTCATTGCCAATTGGCGAAACAACCAGCTTTTTAATGGCAATAT
  v50         v60         v70         v80         v90         v100
CGTATTTTACTAATCTCCACCTTCCTCGTTAACATCGAGCTTCAGATACAACACA
        v110        v120        v130        v140        v150
CAGATCCCTGATTCTTCTTCTATAAACCAAAACCTCAGGAACCATAAAAAAAAAA
  v160        v170        v180                    v190        v200
GGGCATCAAAA ATG TTG AAG CTT TCG TGT AAT GTG ACT AAC CAC
            MET LEU LYS LEU SER CYS ASN VAL THR ASN HIS
        v210        v220        v230        v240
TTA CAC ACC TTC TCC TTC TTC TCC GAT TCC TCC CTT TTC ATC
LEU HIS THR PHE SER PHE PHE SER ASP SER SER LEU PHE ILE
        v250        v260        v270        v280
CCG GTT AAT CGC CGT ACC CTC GCC GTC TCG TCT TCT CAG CCA
PRO VAL ASN ARG ARG THR LEU ALA VAL SER SER SER GLN PRO
      v290        v300        v310        v320
AGG AAG CCG GCT TTA GAT CCT CTT CGG GCA GTT ATC TCC GCC
ARG LYS PRO ALA LEU ASP PRO LEU ARG ALA VAL ILE SER ALA
  v330        v340        v350        v360
GAT CAG GGA AGC ATC AGC CCT GTT AAT TCG TGT ACC CCG GCG
ASP GLN GLY SER ILE SER PRO VAL ASN SER CYS THR PRO ALA
v370        v380        v390                    v400        v410
GAT CGG TTC CGA GCT GGT CGA TTG ATG GAA GAT GGT TAT TCT
ASP ARG PHE ARG ALA GLY ARG LEU MET GLU ASP GLY TYR SER
        v420        v430        v440        v450
TAC AAA GAG AAG TTC ATT GTT AGA AGC TAT GAG GTT GGG ATT
TYR LYS GLU LYS PHE ILE VAL ARG SER TYR GLU VAL GLY ILE
        v460        v470        v480        v490
AAC AAA ACC GCC ACC GTC GAG ACA ATT GCT AAT CTC TTA CAG
ASN LYS THR ALA THR VAL GLU THR ILE ALA ASN LEU LEU GLN
      v500        v510        v520        v530
GAG GTG GCA TGT AAC CAT GTT CAG AAG TGT GGA TTC TCG ACC
GLU VAL ALA CYS ASN HIS VAL GLN LYS CYS GLY PHE SER THR
  v540        v550        v560        v570
GAT GGA TTT GCC ACA ACA CTC ACC ATG AGG AAA TTG CAT CTC
ASP GLY PHE ALA THR THR LEU THR MET ARG LYS LEU HIS LEU
v580        v590        v600                    v610        v620
ATA TGG GTC ACT GCA AGA ATG CAC ATT GAG ATC TAC AAG TAC
ILE TRP VAL THR ALA ARG MET HIS ILE GLU ILE TYR LYS TYR
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Fig. 1 Sheet 1

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          v630                v640                v650                v660
CCA GCT TGG AGT GAT GTT GTT GAG ATA GAG ACA TGG TGC CAG
PRO ALA TRP SER ASP VAL VAL GLU ILE GLU THR TRP CYS GLN

          v670                v680                v690                v700
AGT GAA GGA AGG ATT GGA ACG AGA CGT GAT TGG ATT CTA AGG
SER GLU GLY ARG ILE GLY THR ARG ARG ASP TRP ILE LEU ARG

     v710                v720                v730                v740
GAC TCT GCT ACA AAT GAA GTT ATT GGG CGT GCT ACA AGC AAG
ASP SER ALA THR ASN GLU VAL ILE GLY ARG ALA THR SER LYS

  v750                v760                v770                v780
TGG GTG ATG ATG AAC CAA GAC ACA AGG CGG CTT CAA AGA GTT
TRP VAL MET MET ASN GLN ASP THR ARG ARG LEU GLN ARG VAL

v790                v800                v810                v820                v830
ACA GAT GAA GTT CGG GAC GAG TAC TTG GTT TTC TGT CCT CGA
THR ASP GLU VAL ARG ASP GLU TYR LEU VAL PHE CYS PRO ARG

          v840                v850                v860                v870
GAA CCC AGA CTA GCG TTT CCA GAA GAG AAC AAT AGC AGC TTA
GLU PRO ARG LEU ALA PHE PRO GLU GLU ASN ASN SER SER LEU

          v880                v890                v900                v910
AAG AAA ATC CCA AAA CTA GAA GAT CCA GCT CAG TAT TCT ATG
LYS LYS ILE PRO LYS LEU GLU ASP PRO ALA GLN TYR SER MET

     v920                v930                v940                v950
CTA GAG CTT AAG CCT CGG CGA GCT GAT CTG GAC ATG AAC CAG
LEU GLU LEU LYS PRO ARG ARG ALA ASP LEU ASP MET ASN GLN

  v960                v970                v980                v990
CAC GTG AAT AAC GTC ACC TAC ATT GGA TGG GTG CTT GAG AGC
HIS VAL ASN ASN VAL THR TYR ILE GLY TRP VAL LEU GLU SER

v1000               v1010               v1020               v1030               v1040
ATA CCT CAA GAA ATC ATT GAT ACG CAT GAG CTT CAA GTT ATA
ILE PRO GLN GLU ILE ILE ASP THR HIS GLU LEU GLN VAL ILE

          v1050               v1060               v1070               v1080
ACT CTA GAT TAC AGA AGA GAA TGC CAG CAA GAT GAC ATT GTA
THR LEU ASP TYR ARG ARG GLU CYS GLN GLN ASP ASP ILE VAL

          v1090               v1100               v1110               v1120
GAT TCA CTC ACC ACC TCT GAA ATC CCT GAC GAC CCG ATC TCA
ASP SER LEU THR THR SER GLU ILE PRO ASP ASP PRO ILE SER

     v1130               v1140               v1150               v1160
AAG CTT ACC GGG ACC AAC GGA TCT GCC ACG TCA AGC ATA CAA
LYS LEU THR GLY THR ASN GLY SER ALA THR SER SER ILE GLN

  v1170               v1180               v1190               v1200
GGA CAC AAT GAG AGC CAA TTC TTG CAT ATG CTG AGG TTG TCA
GLY HIS ASN GLU SER GLN PHE LEU HIS MET LEU ARG LEU SER
```

Fig. 1 Sheet 2

```
v1210          v1220          v1230          v1240          v1250
GAA AAT GGC CAG GAG ATC AAT CGT GGA AGA ACA CAA TGG AGA
GLU ASN GLY GLN GLU ILE ASN ARG GLY ARG THR GLN TRP ARG

          v1260          v1270     v1280     v1290     v1300
AAG AAA TCC TCA CGA TGATTTTCTTATATGCTTCTCCAGTGTTGGTGAAG
LYS LYS SER SER ARG TER

        v1310     v1320     v1330     v1340     v1350
TTCTTGCTTCACGTTTATAGTTTTATTGTGTCTTTTGATCAGATCTGTTGTGGGG

   v1360     v1370     v1380     v1390     v1400     v1410
TTAGATGGTACTGGATTATTTGTGTAGTGTGGCGTCTGTGTTGTTCGTTTTCTGT

        v1420     v1430     v1440     v1450     v1460
CCAAATTTTGGGCTTTAAGCAAAACGTTTCTTAGGTCTGCTTGTAGCTTTTCATA

   v1470     v1480     v1490     v1500     v1510     v1520
AACTAGATTTGTTCATCCAATTATATATGCTGAGTTTCTCTGATGAAACTGGGTA

        v1530     v1540     v1550     v1560     v1570
TGTGATGTTGGAGGCAAAATTTGCACAATTAAAATTTGTATTACATAAGTTTTTT

   v1580     v1590     v1600     v1610     v1620     v1630
GGATAAGCTAAAGTTGTATGCAATGTAATACTTTTTTATTTATTGGAGATTAATA

        v1640     v1650     v1660     v1670     v1680
ATATTTTAAATAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA

   v1690          v1700          v1710
AAAAAAAAAAAAAAAAAACTCGAG
```

Fig.1 Sheet 3

RAPE ACYL-ACP THIOESTERASE CLONE pNL3

```
                    v10         v20         v30         v40
GGCGTCATTGCCAATTGGCGAAACAACCAGCTTTTTAATGGCAATAT

  v50         v60         v70         v80         v90         v100
CGTATTTTACTAATCTCCACCTTCCTCGTTAACATCGAGCTTCAGATACAACACA

        v110        v120        v130        v140        v150
CAGATCCCTGATTCTTCTTCTATAAACCAAAACCTCAGGAACCATAAAAAAAAAA

  v160        v170                v180                v190        v200
GGGCATCAAAA ATG TTG AAG CTT TCG TGT AAT GTG ACT AAC AAC
            MET LEU LYS LEU SER CYS ASN VAL THR ASN ASN

            v210                v220                v230        v240
TTA CAC ACC TTC TCC TTC TTC TCC GAT TCC TCC CTT TTC ATC
LEU HIS THR PHE SER PHE PHE SER ASP SER SER LEU PHE ILE

          v250                v260                v270        v280
CCG GTT AAT CGC CGT ACC ATC GCC GTC TCG TCT TCT CAG CTA
PRO VAL ASN ARG ARG THR ILE ALA VAL SER SER SER GLN LEU

      v290                v300                v310        v320
AGG AAG CCG GCT TTA GAT CCT CTA CGG GCA GTT ATC TCC GCG
ARG LYS PRO ALA LEU ASP PRO LEU ARG ALA VAL ILE SER ALA

  v330                v340                v350        v360
GAT CAG GGA AGC ATC AGC CCT GTT AAT TCG TGT ACA CCG GCG
ASP GLN GLY SER ILE SER PRO VAL ASN SER CYS THR PRO ALA

v370                v380                v390        v400        v410
GAT CGG TTA CGA GCT GGT CGA TTG ATG GAA GAT GGT TAT TCG
ASP ARG LEU ARG ALA GLY ARG LEU MET GLU ASP GLY TYR SER

            v420                v430                v440        v450
TAC AAA GAG AAG TTC ATT GTT AGA AGC TAT GAG GTT GGG ATT
TYR LYS GLU LYS PHE ILE VAL ARG SER TYR GLU VAL GLY ILE

          v460                v470                v480        v490
AAC AAA ACC GCC ACC GTC GAG ACA ATT GCT AAT CTC TTA CAG
ASN LYS THR ALA THR VAL GLU THR ILE ALA ASN LEU LEU GLN

      v500                v510                v520        v530
GAG GTG GCG TGT AAC CAT GTT CAG AAG TGT GGA TTC TCG ACG
GLU VAL ALA CYS ASN HIS VAL GLN LYS CYS GLY PHE SER THR

  v540                v550                v560        v570
GAT GGA TTT GCC ACA ACA CTC ACC ATG AGG AAA TTG CAT CTC
ASP GLY PHE ALA THR THR LEU THR MET ARG LYS LEU HIS LEU

v580                v590                v600        v610        v620
ATA TGG GTC ACT GCA AGA ATG CAC ATT GAG ATC TAC AAA TAT
ILE TRP VAL THR ALA ARG MET HIS ILE GLU ILE TYR LYS TYR
```

Fig. 2 Sheet 1

```
          v630          v640          v650          v660
CCA GCT TGG AGT GAT GTT GTT GAG ATA GAG ACA TGG TGC CAG
Pro Ala Trp Ser Asp Val Val Glu Ile Glu Thr Trp Cys Gln

        v670          v680          v690          v700
AGT GAA GGA AGG ATT GGA ACG AGA CGT GAT TGG ATT CTA AGG
Ser Glu Gly Arg Ile Gly Thr Arg Arg Asp Trp Ile Leu Arg

     v710          v720          v730          v740
GAC TCT GCT ACA AAT GAA GTT ATT GGA CGT GCT ACA AGC AAG
Asp Ser Ala Thr Asn Glu Val Ile Gly Arg Ala Thr Ser Lys

   v750          v760          v770          v780
TGG GTG ATG ATG AAC CAA GAC ACA AGG CGG CTT CAA AGA GTT
Trp Val Met Met Asn Gln Asp Thr Arg Arg Leu Gln Arg Val

v790          v800          v810          v820          v830
ACA GAT GAA GTT CGG GAC GAG TAC TTG GTT TTC TGT CCT CGA
Thr Asp Glu Val Arg Asp Glu Tyr Leu Val Phe Cys Pro Arg

          v840          v850          v860          v870
GAA CCC AGA CTA GCG TTT CCA GAA GAG AAC AAT AGC AGC TTA
Glu Pro Arg Leu Ala Phe Pro Glu Glu Asn Asn Ser Ser Leu

        v880          v890          v900          v910
AAG AAA ATC CCA AAA CTA GAA GAT CCA GCT CAG TAT TCT ATG
Lys Lys Ile Pro Lys Leu Glu Asp Pro Ala Gln Tyr Ser Met
```

Fig.2.
Sheet 2

```
     v920          v930          v940          v950
CTA GAG CTT AAG CCT CGG CGA GCT GAT CTG GAC ATG AAC CAG
Leu Glu Leu Lys Pro Arg Arg Ala Asp Leu Asp Met Asn Gln

  v960          v970          v980          v990
CAC GTG AAT AAC GTC ACC TAC ATC GGA TGG GTG CTT GAG AGC
His Val Asn Asn Val Thr Tyr Ile Gly Trp Val Leu Glu Ser

v1000         v1010         v1020         v1030         v1040
ATA CCT CAA GAA ATC ATT GAT ACG CAT GAG CTT CAA GTT ATA
Ile Pro Gln Glu Ile Ile Asp Thr His Glu Leu Gln Val Ile

          v1050         v1060         v1070         v1080
ACT CTA GAT TAC AGA AGA GAA TGC CAG CAA GAT GAC ATT GTA
Thr Leu Asp Tyr Arg Arg Glu Cys Gln Gln Asp Asp Ile Val

        v1090         v1100         v1110         v1120
GAT TCA CTC ACC ACC TCT GAA ATC CCT GAC GAC CCG ATC TCA
Asp Ser Leu Thr Thr Ser Glu Ile Pro Asp Asp Pro Ile Ser

     v1130         v1140         v1150         v1160
AAG TTT ACC GGG ACC AAC GGA TCT GCC ATG TCA AGC ATA CAA
Lys Phe Thr Gly Thr Asn Gly Ser Ala Met Ser Ser Ile Gln

  v1170         v1180         v1190         v1200
GGA CAC AAT GAG AGC CAG TTC TTG CAT ATG CTG AGG TTG TCA
Gly His Asn Glu Ser Gln Phe Leu His Met Leu Arg Leu Ser
```

Fig. 2, Sheet 3

```
v1210          v1220          v1230          v1240          v1250
GAA AAT GGC CAG GAG ATC AAT CGT GGG AGA ACA CAA TGG AGA
GLU ASN GLY GLN GLU ILE ASN ARG GLY ARG THR GLN TRP ARG

          v1260          v1270     v1280     v1290     v1300
AAG AAA TCC TCA CGA TGATTTTCTTATATGCTTCTCCAGTGTTGGTGAAG
LYS LYS SER SER ARG TER

         v1310     v1320     v1330     v1340     v1350
TTCTTGCTTCACGTTTATAGTTTTATTGTCTCTTTTGATCAGATCTATTGTGGGG

   v1360     v1370     v1380     v1390     v1400     v1410
TTTAGATGGTACTGGATTATTTGTGTAGTGTGGCGTCTGTGTTATTCGTTTTCTG

         v1420     v1430     v1440     v1450     v1460
TCCAAATTTTGGGCTTTAAGCAAAACGTTTCTTAGGTCTTAATCTTTTGATCCGC

   v1470     v1480     v1490     v1500     v1510     v1520
TTGTATCTTTTCATAAACTAGATTTTTTCATCCAATTATATATGCTGAGTTTCTC

         v1530     v1540     v1550     v1560     v1570
TTAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA

   v1580
AAAACTCGAG
```

Fig. 2, Sheet 4

RAPE ACYL-ACP THIOESTERASE

```
NL2  GGCGTCATTGCCAATTGGCGAAACAACCAGCTTTTTAATGGCAATATCGTATTTTACTAATCTCCACCTTCCTCGTTAAC   80
NL3  ----------------------------------------------------------------------------------   80

NL2  ATCGAGCTTCAGATACAACACACAGATCCCTGATTCTTCTTCTATAAACCAAAACCTCAGGAACCATAAAAAAAAAAGGG  160
NL3  ----------------------------------------------------------------------------------  160

NL2  CATCAAAAATGTTGAAGCTTTCGTGTAATGTGACTAACCACTTACACACCTTCTCCTTCTTCTCCGATTCCTCCCTTTTC  240
NL3  -----------------------------------A----------------------------------------------  240

NL2  ATCCCGGTTAATCGCCGTACCCTCGCCGTCTCGTCTTCTCAGCCAAGGAAGCCGGCTTTAGATCCTCTTCGGGCAGTTAT  320
NL3  --------------------A---------------------T------------------------A-------------  320

NL2  CTCCGCCGATCAGGGAAGCATCAGCCCTGTTAATTCGTGTACCCCGGCGGATCGGTTCCGAGCTGGTCGATTGATGGAAG  400
NL3  -------G----------------------------------A--------------A----------------------  400

NL2  ATGGTTATTCTTACAAAGAGAAGTTCATTGTTAGAAGCTATGAGGTTGGGATTAACAAAACCGCCACCGTCGAGACAATT  480
NL3  ----------G---------------------------------------------------------------------  480

NL2  GCTAATCTCTTACAGGAGGTGGCATGTAACCATGTTCAGAAGTGTGGATTCTCGACCGATGGATTTGCCACAACACTCAC  560
NL3  ----------------------G---------------------------------G----------------------  560

NL2  CATGAGGAAATTGCATCTCATATGGGTCACTGCAAGAATGCACATTGAGATCTACAAGTACCCAGCTTGGAGTGATGTTG  640
NL3  ------------------------------------------------------A--T--------------------  640

NL2  TTGAGATAGAGACATGGTGCCAGAGTGAAGGAAGGATTGGAACGAGACGTGATTGGATTCTAAGGGACTCTGCTACAAAT  720
NL3  ----------------------------------------------------------------------------------  720

NL2  GAAGTTATTGGGCGTGCTACAAGCAAGTGGGTGATGATGAACCAAGACACAAGGCGGCTTCAAAGAGTTACAGATGAAGT  800
NL3  -----------A----------------------------------------------------------------------  800

NL2  TCGGGACGAGTACTTGGTTTTCTGTCCTCGAGAACCCAGACTAGCGTTTCCAGAAGAGAACAATAGCAGCTTAAAGAAAA  880
NL3  ----------------------------------------------------------------------------------  880
```

Fig. 3, Sheet 1

```
NL2  TCCCAAAACTAGAAGATCCAGCTCAGTATTCTATGCTAGAGCTTAAGCCTCGGCGAGCTGATCTGGACATGAACCAGCAC   960
NL3  ------------------------------------------------------------------------------   960

NL2  GTGAATAACGTCACCTACATTGGATGGGTGCTTGAGAGCATACCTCAAGAAATCATTGATACGCATGAGCTTCAAGTTAT  1040
NL3  --------------------C-------------------------------------------------------  1040

NL2  AACTCTAGATTACAGAAGAGAATGCCAGCAAGATGACATTGTAGATTCACTCACCACCTCTGAAATCCCTGACGACCCGA  1120
NL3  ------------------------------------------------------------------------------  1120

NL2  TCTCAAAGCTTACCGGGACCAACGGATCTGCCACGTCAAGCATACAAGGACACAATGAGAGCCAATTCTTGCATATGCTG  1200
NL3  --------T-------------------------T-------------------------------G---------  1200

NL2  AGGTTGTCAGAAAATGGCCAGGAGATCAATCGTGGAAGAACACAATGGAGAAAGAAATCCTCACGATGATTTTCTTATAT  1280
NL3  ------------------------------------G-----------------------------------------  1280

NL2  GCTTCTCCAGTGTTGGTGAAGTTCTTGCTTCACGTTTATAGTTTTATTGTGTCTTTTGATCAGATCTGTTGTGGGGTT-A  1359
NL3  ----------------------------------------------C----------------A----------T-  1360

NL2  GATGGTACTGGATTATTTGTGTAGTGTGGCGTCTGTGTTGTTCGTTTTCTGTCCAAATTTTGGGCTTTAAGCAAAACGTT  1439
NL3  ----------------------------------------A-------------------------------------  1440

NL2  TCTTAGGTCT--------------GCTTGTAGCTTTTCATAAACTAGATTTGTTCATCCAATTATATATGCTGAGTTTCT  1505
NL3  ----------TAATCTTTTGATCC--------T-------------------T---------------------------  1520

NL2  CTGATGAAACTGGGTATGTGATGTTGGAGGCAAAATTTGCACAATTAAAATTTGTATTACATAAGTTTTTTGGATAAGCT  1585
NL3  --TAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAACTCGAG              1586

NL2  AAAGTTGTATGCAATGTAATACTTTTTTATTTATTGGAGATTAATAATATTTTAAATAAAAAAAAAAAAAAAAAAAAAA  1665

NL2  AAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAACTCGAG                                    1710
```

Fig. 3, Sheet 2

RAPE ACYL-ACP THIOESTERASE

```
NL2   MLKLSCNVTNHLHTFSFFSDSSLFIPVNRRTLAVSSSQPRKPALDPLRAV   50
NL3   ----------N----------I------L----------              50

NL2   ISADQGSISPVNSCTPADRFRAGRLMEDGYSYKEKFIVRSYEVGINKTAT  100
NL3   ----------L----------                                100

NL2   VETIANLLQEVACNHVQKCGFSTDGFATTLTMRKLHLIWVTARMHIEIYK  150
NL3   ----------                                          150

NL2   YPAWSDVVEIETWCQSEGRIGTRRDWILRDSATNEVIGRATSKWVMMNQD  200
NL3   ----------                                          200

NL2   TRRLQRVTDEVRDEYLVFCPREPRLAFPEENNSSLKKIPKLEDPAQYSML  250
NL3   ----------                                          250

NL2   ELKPRRADLDMNQHVNNVTYIGWVLESIPQEIIDTHELQVITLDYRRECQ  300
NL3   ----------                                          300

NL2   QDDIVDSLTTSEIPDDPISKLTGTNGSATSSIQGHNESQFLHMLRLSENG  350
NL3   ----------F----------M----------                    350

NL2   QEINRGRTQWRKKSSR                                    366
NL3   ----------                                          366
```

Fig.4

RAPE THIOESTERASE PEPTIDE SEQUENCES

ENDOPROTEINASE LYS-C

PEPTIDE 26	F I V R S Y E V G I N K

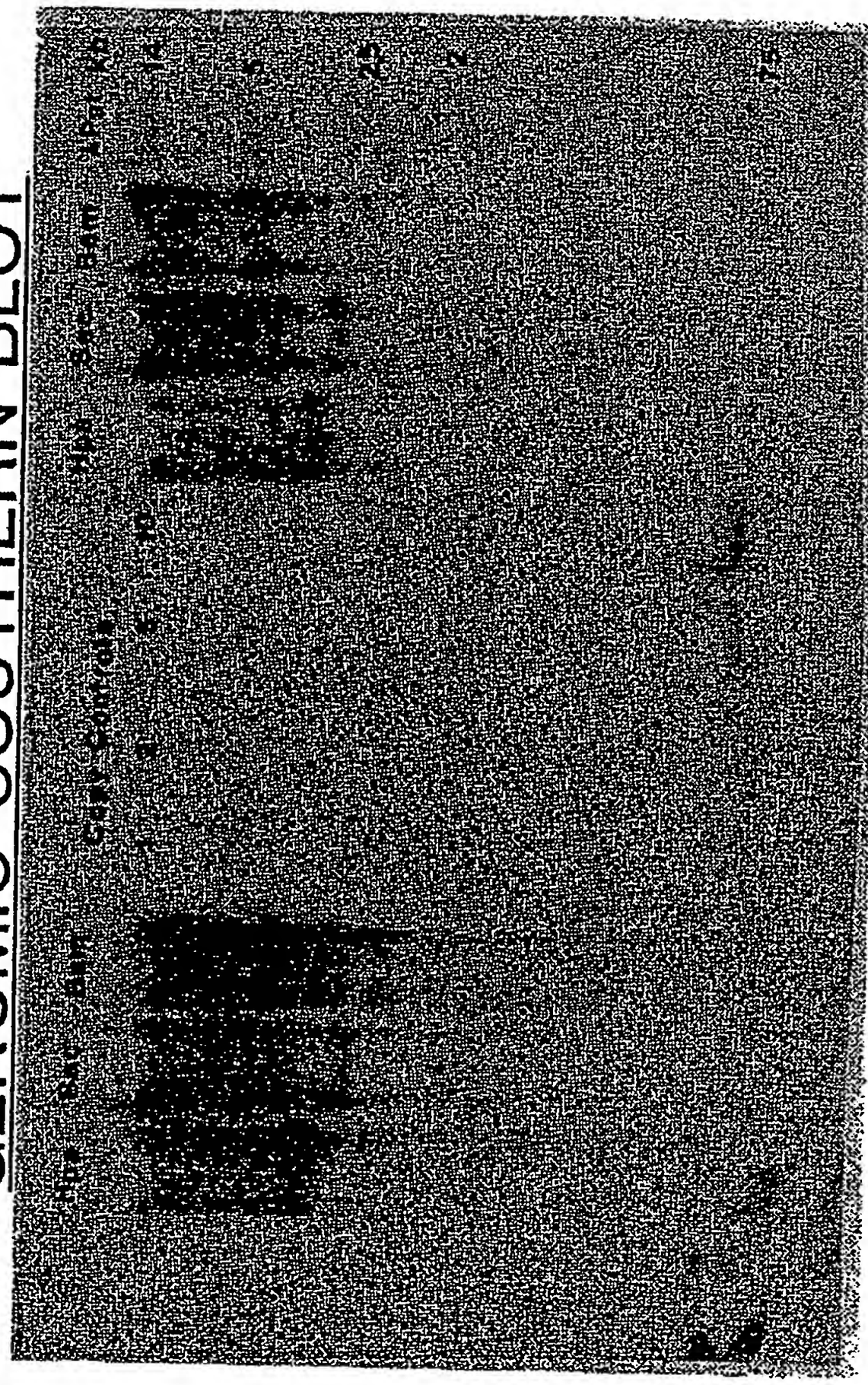
TRYPSIN

PEPTIDE 15	L S [G D] G Q E I N R
PEPTIDE 30	L A F P E E N N [R]
PEPTIDE 34	V M M N Q D T R
PEPTIDE 39	D W I L R

Fig. 5

Fig.6

RAPE THIOESTERASE
GENOMIC SOUTHERN BLOT



Hpal - 12 copies per haploid genome
" "
" "
SacI - 17 copies
" "
" "
BamHI - 16 copies
" "
" "

INTERNATIONAL SEARCH REPORT

International Application No PCT/GB 93/00432

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl. 5 C12N15/55; C12N15/82; C12N9/16; C12N5/10
C12N1/21; A01H1/00

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
Int.Cl. 5	C12N ; A01H

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category °	Citation of Document,[11] with indication, where appropriate, of the relevant passages[12]	Relevant to Claim No.[13]
X	PLANT LIPID BIOCHEMISTRY, STRUCTURE AND UTILIZATION; NINTH INTERNATIONAL SYMPOSIUM ON PLANT LIPIDS, KENT, ENGLAND, UK, JULY 8-13, 1990. 1990, PORTLAND PRESS LTD., LONDON, ENGLAND pages 157 - 159 HELLYER, A., ET AL. 'Acyl-acyl-carrier protein thioesterase from oil seed rape purifiaction and characterization'	7,8
Y	see the whole document	1-6,9-16

Y	WO,A,9 116 421 (CALGENE) 31 October 1991 see the whole document	1-6,9-16

	-/--	

° Special categories of cited documents :[10]

"A" document defining the general state of the art which is not considered to be of particular relevance

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"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

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"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
27 MAY 1993	14 -06- 1993
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	MADDOX A.D.

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category°	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
Y	BIOTECHNOLOGY vol. 10, no. 1, January 1992, NEW YORK US page 59 'Advances in gene technology: Feeding the world in the 21st century' see right column, line 6 - line 10 & MIAMI SHORT REPORTS.1992 MIAMI WINTER SYMP., JAN 19-24, 1992. vol. 2, 1992, page 102 VOELKER, T.A., ET AL 'Engineering laurate production in oilseeds'	1-6,9-16
P,X	WO,A,9 211 373 (DU PONT) 9 July 1992 see example 5	1-6,9,10
P,X	WO,A,9 220 236 (CALGENE) 26 November 1992 see page 58, line 30 - line 36; figure 6	1,3,4,7, 9,10

ANNEX TO THE INTERNATIONAL SEARCH REPORT
ON INTERNATIONAL PATENT APPLICATION NO. GB 9300432
SA 70674

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.
The members are as contained in the European Patent Office EDP file on
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Patent document cited in search report	Publication date	Patent family member(s)	Publication date
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WO-A-9211373	09-07-92	AU-A- 9116191	22-07-92
WO-A-9220236	26-11-92	None	